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WHEN IS THE SEX RATIO BIASED IN SOCIAL SPIDERS?: CHROMOSOME STUDIES OF EMBRYOS AND MALE MEIOSIS IN *ANELOSIMUS* SPECIES (ARANEAE, THERIDIIDAE)

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Abstract. Embryo chromosome preparations of four species of social spiders of the genus *Anelosimus* show that the two species known or suspected to form permanent, multigenerational colonies, *A. eximius* and *A. domingo*, have a highly female-biased primary sex ratio. *Anelosimus jucundus* and *A. studiosus*, on the other hand, are shown to produce an even number of males and females. The magnitude of the bias of *A. eximius* embryos is similar to that reported for young preadult spiders of this species, therefore ruling out differential mortality of juveniles as the cause of this species' sex ratio bias. Chromosome counts of nuclei in second division of *A. eximius* male meiosis indicate that nuclei destined to yield sons and daughters are produced in equal numbers. Therefore, the sex ratio biasing mechanism in this species must act after male meiosis and before egg laying. The question of how early the sex ratio bias arises still needs to be resolved in other social spiders. We discuss some methodological and theoretical complications associated with measuring sex ratios at different stages of the life cycle and present a fast and reliable technique to obtain embryo chromosome preparations.

The occurrence of highly female-biased sex ratios among adults of several species of social spiders has been known since the 1960's (Buskirk 1981), but there has been little study of exactly when in the spiders' life cycle the sex ratio bias arises. Knowledge of the timing of the sex ratio bias is important on at least two accounts: first, from an evolutionary point of view, it would help us determine whether differential parental investment is involved in biasing the sex ratio; and, second, from a physiological point of view, it would bring us closer to identifying the mechanism by which the sex ratio bias is accomplished.

Fisher's principle (Fisher 1930) states that, at equilibrium, the total parental investment in offspring of each sex should be equal. Departures from this equilibrium should bring about selection to restore an even sex ratio because individuals of the rare sex would have a reproductive advantage. Exceptions to Fisher's sex ratio principle have been pointed out by Hamilton (1967), who first noted that biased sex ratios can evolve when the assumption of panmixia, implicit in Fisher's argument, is violated. Parasitic and fig wasps (e.g., Werren 1980; Waage 1982; Herre 1985) and hummingbird-flower mites (Wilson and Colwell 1981) are notable examples. As first noted by Avilés (1983, 1986), social spiders appear to represent another case in which Fisher's principle has been violated. In most social spiders, however, this violation has not been confirmed because their sex ratio has been measured late enough in the spider's life cycle (usually among adults) that higher male mortality during the preadult or adult instars cannot be ruled out as the cause of their sex ratio bias. As noted by several authors (Leigh 1970; Charnov 1982; Trivers 1985), if biased sex ratios are due solely to mortality occurring after the end of the period of parental investment then Fisher's principle is not violated. In three species, *Anelosimus eximius* Keyserling, *Achaearanea wau* Levi and *Stegodyphus dumicola* Pocock, there is indirect evidence that more females are actually being

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produced. This evidence comes either from young-preadult sex ratios estimated in isolated natural colonies (Avilés 1983, 1986) or from preadult/adult sex ratios measured among individuals raised under eontrolled conditions, either from egg sacs (Vollrath 1986b; Lubin and Crozier 1985; Lubin in press) or from a colony maintained in the laboratory (Seibt and Wickler 1988). These measurements provide evidence of an early bias, but may still be affected by mortality during the juvenile instars.

In this paper we present a eytogenetic method that makes it possible to directly determine the sex of a developing embryo and, therefore, to measure the sex ratio before mortality becomes a factor. We apply this method to four species of the genus *Anelosimus* Simon (Levi 1956, 1963) present in Ecuador. Two of these, *A. eximius* and *A. domingo* Levi, are among the most social in the genus, with spiders that cooperate in prey capture and brood care and share a permanent communal nest generation after generation (*A. eximius* referenees: Brach 1975; Tapia & De Vries 1980; Christenson 1984; *A. domingo* references: Levi & Smith 1982; Rypstra & Tirey 1989). The other two, *A. jucundus* O. P.- Cambridge and *A. studiosus* Hentz, on the other hand, are known to exhibit a less advanced form of sociality where the offspring of a single female remain together for the early part of their life cyele but disperse before reaching adulthood (Brach 1977; Nentwig & Christenson 1986). Unlike *A. eximius*, *A. jucundus* and *A. studiosus* have been reported to have 1:1 preadult or adult sex ratios (Fowler & Levi 1979; Nentwig & Christenson 1986; Vollrath 1986b reared offspring from two *A. jucundus* sacs and obtained an even sex ratio). *Anelosimus domingo* sex ratios have not been previously reported.

Should it be eonfirmed that the embryo sex ratios are biased, the next question to be answered is what is the mechanism by which the sex ratio bias arises. This question is of special interest in spiders because, unlike haplodiploid organisms that constitute most other known cases of extreme sex ratio biases (Hamilton 1967), spiders are diploid organisms with chromosomal sex determination and therefore lack the opportunity to bias the sex ratio by choosing whether or not to fertilize the egg. In this paper we examine the possibility that the earliest acting mechanism, a bias in male meiosis leading to the excess produetion of sperm destined to yield daughters, may occur in *A. eximius*.

MATERIALS AND METHODS

Chromosome preparations were obtained from embryos and males collected in Ecuador from naturally occurring colonies of the four *Anelosimus* species. The sex of an embryo, or whether a nucleus in a spider testis is going to become a male- or a female-producing spermatozoid, can be determined cytologically thanks to the difference in chromosome number between male and female spiders. The most common mechanism of sex determination in spiders involves two pairs of X chromosomes, with the two members of each pair present in females ($X_1X_1X_2X_2$) and only one in males (X_1X_2O) (White 1973). The sex of an individual egg or of a developing spermatozoid can therefore be simply determined by obtaining its chromosome count.

Anelosimus eximius egg sacs were collected in July 1988 from two colonies, approximately 1.5 km apart, near the Recinto A. Perez Intriago, Km 113, Quito - Pto. Quito road (0°6′N:79°5′W). *Anelosimus* egg sacs were also collected near Perez Intriago in June 1989 from one colony found in a forest pocket near the Silanche river. *Anelosimus jucundus* sacs were collected from two colonies in Crucita, Manabí (0°52′S:80°33′W), in August 1988 and *A. studiosus* near Calderón, Pichincha (0°6′S:78°27′W), in July 1989. Males of the four species were collected from the same sites as the egg sacs, except that in addition one male of *A. studiosus* was collected from El Tingo, Pichincha (0°17′S:78°27′W). Egg sacs and males were brought alive to the laboratory where the preparations were made.

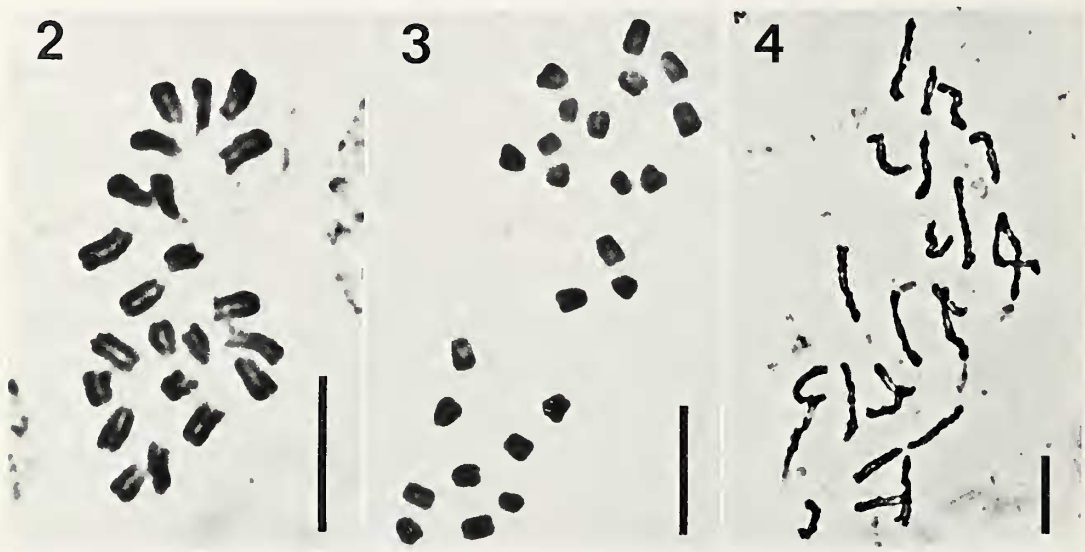
The technique we developed to obtain chromosome preparations from individual eggs is described in the Appendix. Basically, it is much like typical acetic squash methods (e.g., Darlington & La Cour 1975) which stain then squash, except that it stains after squashing so as to yield much superior squashing. The stage of development of the eggs at the moment the sacs were collected was not known. However, we found that good preparations can be obtained from a wide range of stages, from very young embryos whose limb buds are just beginning to appear to older ones with well formed buds prior to the development of a euticular covering. Once the cuticle has been formed, squashing is not as good and there are fewer dividing cells. All eggs in the *A. domingo* and *A. eximius* sacs that appeared to be developing normally were squashed (one to three eggs in the first three *A. eximius* sacs

Figure 1.—Metaphase I nucleus in *A. eximius* male meiosis. Note 10 acrocentric bivalents and two X chromosomes. Scale bar = 5 μm.

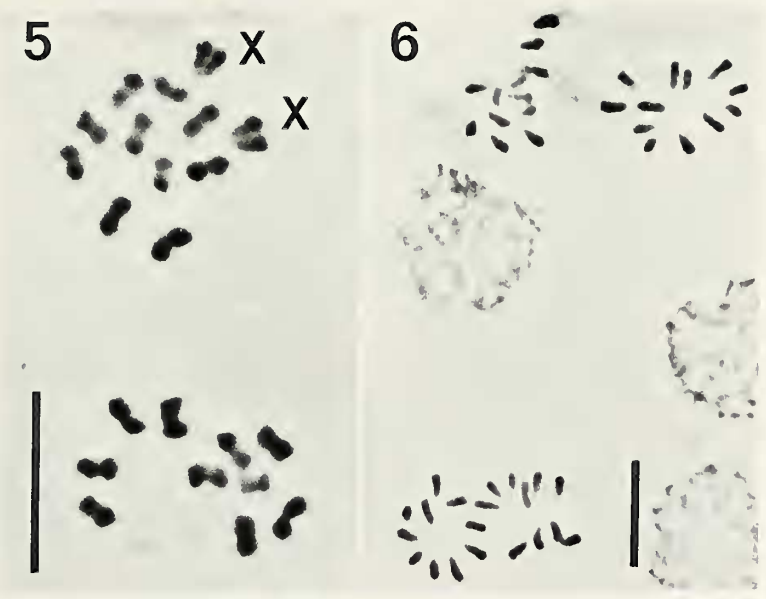
were lost due to mishandling). In *A. studiosus*, a random sample of around 30 eggs per sac was chosen. In *A. jucundus*, a similar sample size was chosen, though with the two egg sacs unevenly represented due to differences in their developmental stage. Sampling in *A. jucundus* could not be entirely random because the eggs in a sac were found to be widely asynchronous in their development and the ones that were obviously too old to yield good preparations were not squashed.

Chromosomes were counted under 1000X using oil immersion. In the embryo preparations, nuclei with countable chromosomes were sought on the microscope slide until at least three were found with the same chromosome count of either 22 (the male diploid complement) or 24 (female); if three nuclei with 22 or 24 chromosomes could not be found, the egg was deemed unscorable. With few exceptions (see Table 1), around 90% of the preparations for a given sac could be scored. Since it is reasonable to suppose that the eggs scored were a random sample of the eggs squashed and since, with the only exception of *A. jucundus*, the eggs squashed were all or a random sample of those in a sac, the sex ratios obtained provide a direct estimation of the primary sex ratios of the species studied. Fewer than 90% percent of the preparations in the *A. domingo* sacs 1 and 4 could be scored because, when the sacs were first opened, the eggs were still too young to yield enough cells for scoring. After the first one or two eggs, these sacs were closed and the preparations continued at a later date. In the case of *A. jucundus*, because of the asynchrony in the development of the eggs in a sac, some were too young or too old to yield good preparations.

Chromosome preparations from testes were obtained by using one of three techniques: (a) Feulgen, as done by Maddison (1982), (b) squashing after staining with aceto-orcein, or (c) the same technique as that described for the eggs. One *A. domingo*, nine *A. eximius*, two *A. jucundus* and three *A. studiosus* males were examined



Figures 2–4.—*A. eximius* embryo chromosomes: 2, male embryo (22 chromosomes); 3, 4, female embryos (24 chromosomes). Scale bar = 10 μm.



Figures 5, 6.—Second division nuclei of *A. eximius* male meiosis: 5, two Metaphase II nuclei; top has 12 chromosomes, bottom, 10; 6, two pairs of early Telophase II nuclei, top has 12 chromosomes each member of the pair, bottom, 10. Scale bar = 10 μm.

to confirm the chromosome complements of the four species. The nine *A. eximius* males were further examined to investigate the possibility of a bias in male meiosis leading to the overproduction of XX-bearing spermatids. *A. eximius* slides were scanned systematically and all scorable nuclei found, scored. Counts were made of either each second metaphase nucleus (Fig. 5) or of each pair of early second telophase nuclei, which nearly always occurred together (Fig. 6). Confidence limits for the proportions at the 95% level were obtained from binomial confidence

Table 1.—Number of female and male embryos present in individual egg sacs of four species of the genus *Anelosimus*, as determined by their chromosome count: females, 24 chromosomes, and males, 22. The total number of eggs in a sac, the number squashed for chromosomes and, of those, the percent that yielded preparations whose chromosome count could be scored are given in columns 3–5.

		Eggs				
Species	Sac no.	Total	Squashed	% scorable	Females	Males
A. domingo	1	16	16	81	12	1
	2	13	13	92	11	1
	3	14	14	93	12	1
	4	15	15	73	10	1
A. eximius	1	51	43	91	35	4
	2	45	44	89	37	2
	3	53	50	88	39	5
	4	51	51	92	42	5
A. jucundus	1	73	16	75	6	6
	2	71	43	63	14	13
A. studiosus	1	39	31	94	15	14
	2	47	30	83	13	12

Table 2.—Primary sex ratio of four species of the genus *Anelosimus* reported as the proportion of male embryos contained in 2–4 egg sacs per species.

Species	No. sacs	No. eggs	Males	Females	Proportion males	95% c.i.
A. domingo	4	50	4	45	0.08	0.02–0.19
A. eximius	4	172	16	153	0.09	0.05–0.14
A. jucundus	2	39	19	20	0.49	0.33–0.66
A. studiosus	2	54	26	28	0.48	0.34–0.62

interval graphs, as presented by Remington and Schork (1985).

RESULTS

Chromosome complement of *Anelosimus* spp.—Chromosome counts in male meiosis showed that the male diploid complement in all four species of *Anelosimus* examined is 20 autosomes + XXO (Fig. 1), the typical complement for the family Theridiidae (Suzuki 1954). Males, therefore, have 22 chromosomes, and females should have 24 chromosomes. As expected, developing eggs were found to have either 22 or 24 chromosomes in all four species examined (Figs. 2–4). The 20 autosomes, as well as the two X chromosomes, are acrocentrics. One male of *A. studiosus* showed an extra chromosome, possibly a supernumerary.

Sex ratio of eggs.—*Anelosimus jucundus* and *A. studiosus* were found to have an even primary sex ratio (Table 2), while the other two species, *A. domingo* and *A. eximius*, were found to have highly female biased primary sex ratios. *Anelosimus domingo* egg sacs contained a single male out of 11 to 13 eggs and *A. eximius* sacs contained from 2 to 5 males out of 39 to 47 eggs (Table 1). The proportion of males found among embryos from the four egg sacs of *A. domingo* is 0.08 and of *A. eximius*, 0.09 (Table 2).

All the eggs in the *A. domingo* sacs and in three of the *A. eximius* sacs were found to be developing normally. One of the *A. eximius* sacs (#1) contained 1 dried up egg and 6 egg shells, most likely the remains of eggs eaten up by two hymenopteran parasitic larvae found in the sac.

Is the sex ratio biased by male meiosis?.—By the second division of male meiosis, nuclei destined to become male-producing sperm have 10 chromosomes, those destined to become female-producing sperm have 12. At telophase II (Fig. 6), the ratio of male-producing to female-producing nuclei was found to be very close to 1:1 (ratio 0.49, $N = 105$ pairs, 95% confidence interval = 0.34–0.59), showing that male meiosis is not biased. In the earlier stage of metaphase II (Fig. 5) we obtained a slightly biased ratio (ratio 0.39, $N = 57$, 95% confidence interval = 0.270.53) which however was not significantly different from 1:1 ($p > 0.11$, by an exact two-tailed test based on the binomial probabilities). The slight bias observed in metaphase II is probably due to sampling error given that the number of nuclei scored at this phase is smaller (57 vs. 105) and that at the later telophase II stage the two types of nuclei occur in even numbers.

DISCUSSION

This study shows that the sex ratio among developing embryos of two of the most social species of the genus *Anelosimus*, *A. eximius* and *A. domingo*, is highly female biased. The bias in *A. eximius* is of the same magnitude as that previously reported from preadult individuals of this species (Avilés 1986 and unpublished data) and from individuals raised from eggs (Vollrath 1986b). This shows that differential mortality of the sexes during the juvenile instars is not responsible for the sex ratio bias and that the bias results from an overproduction of females by the time the eggs are laid. The sex ratio is therefore biased throughout the life cycle, and parental investment in *A. eximius*, as in *A. domingo*, is heavily skewed towards females. This removes any doubts that this bias represents a violation of Fisher's principle, on the one hand, and pushes back the moment at which the sex ratio biasing mechanism must act to the period previous to egg laying. In the other two species, *A. jucundus* and *A. studiosus*, even primary sex ratios were found.

The difference in sex ratio between *A. jucundus* and *A. studiosus*, on the one hand, and *A. eximius* and *A. domingo*, on the other, is consistent with what is known about the mating system and pop-

ulation structure of these species. *Anelosimus jucundus* and *A. studiosus* form colonies that disintegrate before its members (usually the progeny of a single female) reach adulthood (Brach 1977; Nentwig & Christenson 1986; Avilés unpublished). Therefore, mating in these species takes place among individuals from the population at large and, as observed, an even sex ratio is expected. The colonies of *A. eximius*, on the other hand, as a consequence of permanent sociality, constitute isolated lineages whose members reproduce by inbreeding generation after generation (Overal & Ferreira 1982; Vollrath 1982; Smith 1982; Avilés 1983, 1986). According to a model proposed (Avilés 1983, 1986, in prep.), the isolated descent of many small lineages and their rapid turnover rate would bring about the conditions under which selection at the colony level can override fisherian selection within colonies, making female-biased sex ratios evolutionarily stable (see Frank 1987 for a different model). The population structure of the fourth species, *A. domingo*, is not yet known. However, cooperation in this species extends through adulthood (Rypstra & Tirey 1989) and multiple egg-laying females and spiders of all instars occur in the colonies (Avilés unpublished), suggesting that sociality is permanent and that mating takes place within the parental colony, as occurs in *A. eximius*. The strongly female biased sex ratios here reported lead us to predict that the population structure of this species will also be found to be highly subdivided and that the conditions that favor the evolution of female biased sex ratios in *A. eximius* are also present in *A. domingo*.

One of the questions opened by the present study has to do with the mechanism by which such a large overproduction of females is accomplished. This study rules out early death of embryos as a possible mechanism since, with the only exception of one sac, all eggs in the *A. eximius* and *A. domingo* sacs examined were developing normally and almost all were scored. The biasing mechanism must therefore act during the period previous to the deposition of the eggs. Our results also show that a bias in male meiosis, the earliest acting possible mechanism, does not occur in *A. eximius*: by telophase II, nuclei destined to give rise to female- and male-producing sperm occur in equal numbers. This leaves the following stages as possible targets during wich the biasing mechanism can act: the final stages of spermatogenesis, sperm induction, transfer of sperm to the female spermatheca, sperm activation in the spermatheca previous to the fertilization of the eggs, and fertilization itself. Once the eggs have been fertilized, the sex ratio must be already determined, since, as Vollrath (1986b) points out, reabsorption of male zygotes is not likely given that the sperm is added to the eggs as they are being laid. Some form of sperm selection, involving either differential death of sperm, differential activation or sperm competition, appears the most likely mechanism.

The question of when the sex ratio is biased still needs to be resolved in other social spiders. Outside *Anelosimus*, with the already mentioned exceptions of *Achaearanea wau* and *Stegodyphus dumicola* for which rearing experiments have been conducted, sex ratios in other social spiders have only been measured in adults (Jackson & Smith 1978; Riechert et al. 1986), in some combination of adults and subadults (Pain 1964; Kullman et al. 1971) or in some unspecified instar, presumably mature individuals (Darchen 1967; Main 1988; Jacson & Joseph 1973; Seibt & Wickler 1988 for *S. mimosarum* Pavesi). As already mentioned, adult sex ratios are not sufficient evidence that parental investment is biased, and, therefore, that Fisher's principle has been violated. When compared with data taken at earlier stages, adult sex ratio data can nevertheless be useful as evidence that sex specific mortality or migration occurs. However, because in spiders males often mature at least one molt earlier than females, measuring adult sex ratio is much more involved than has been generally regarded. In social spiders, as in any other species in which generations are discrete, either due to seasonality or to recent establishment of the population or colony by just a few founders (Bradoo 1972; Darchen 1978; Lubin & Robinson 1982; Avilés 1986; Main 1988; Seibt & Wickler 1988), the difference in the number of molts makes the proportion of adult males to adult females dependent on the point in the colony life cycle at which the sample is collected (e.g., see fig. 3 of Avilés 1986). This might explain why some authors have obtained some instances of male biased sex ratios (e.g., Vollrath 1986a,b; Riechert et al. 1986, pp. 185, 186; Main 1988, p. 66), the large variability found by most authors (Pain 1964; Bradoo 1975; Jacson & Joseph 1973; Riechert et al. 1986; Vollrath 1986a,b; Seibt & Wickler 1988), and the differences among them.

To solve this problem one might measure adult sex ratio as the proportion of adult males vs. the chronologically equivalent preadult female instar. However, because of the different times the sexes remain in those instars, this estimate is biased against females (female spiders are only temporarily in the preadult instar until molting to adulthood while males of several cohorts accumulate in the adult instar). In general, the persistence time in a particular instar should always be taken into consideration when any two stages in a life cycle are compared by vertical sampling. The measurement that would more fairly compare all males and females of the same cohort would have to count adult males vs. females of the same and all older instars to which they molt while males are still around. However, even this estimate would vary depending upon when in the colony life cycle the sample was collected, if males migrate or die earlier than females. For these reasons, at the moment we do not have good evidence of what the magnitude of the sex ratio bias is in other social spiders or whether it represents a theoretically interesting bias.

Given our current knowledge about the social behavior and population structure of other social spiders, however, our prediction is that their adult sex ratio bias will also be found to result from uneven parental investment. This prediction can now be easily tested using the cytogenetic technique that we present here. It should be noted, however, that in species in which parental care extends beyond conception, measuring sex ratio in embryos will not necessarily tell us all we want to know about parental investment. Whether or not biased at conception, the proportion of male to female offspring, or their relative sizes, may change during the period of parental care as a result of differential mortality or differential allocation of resources. To determine whether this is the case, the sex ratio at the end of the period of parental investment should also be estimated. In social spiders, parental investment can be presumed to end at the instar at which the spiderlings start to participate in the activities of the colony and are therefore less dependent on the parental generation (in *A. eximius*, this occurs at about the same time when males are first recognizable due to their enlarged palpi, Avilés 1986). If this young preadult sex ratio is found to be different from the embryo sex ratio, then parental investment would need to be estimated by integrating the numbers of male and female offspring and the per capita investment in them over the period of parental care. If the sex ratio values are the same, and no size difference between the sexes is obvious, as has been found to be the case in *A. eximius* , then the investment ratio can be estimated from the numerical ratio either among embryos or among young preadults. In studies in which the sex ratio of a large number of colonies needs to be estimated, measuring the preadult sex ratio may be the only feasible alternative. The preadult sex ratio, however, is probably more subject to empirical error because, through time, random mortality or asynchrony in the development times of the sexes would tend to increase the variance of the sex ratio estimate. Aside from being perhaps more accurate, embryo sex ratios have the additional advantage of allowing an assessment of whether there is variation for the sex ratio among the progeny of different females (given that eggs of a clutch are laid together in a sac).

The importance of knowing the primary sex ratio is certainly not limited to social spiders since issues of sex ratio and population structure, sex specific demographic phenomena and sex ratio variation are of general interest in spider biology. The cytogenetic technique that we present here greatly simplifies the estimation of the primary sex ratio in spiders. It not only has obvious advantages over time consuming egg-rearing techniques which risk producing a biased estimate if there is mortality (Fiala 1980), but it also has several advantages over a previously described technique for obtaining spider embryo chromosomes (Matsumoto 1977; Tugmon et al. 1990): it allows reliable preparation of individual eggs and it is fast enough that population studies become feasible. This technique has already been successfully used in mites (M. Kaliszewski pers. comm.) and it can probably be used with equal success in other arthropods (see Crozier 1968 for a more laborious technique used for insect pupae). Widespread use of this technique will make available quantitative sex ratio estimates of a phylogenetically diverse set of species, so that comparative studies to test specific predictions of sex ratio and population structure become possible. Should primary sex ratio biases be confirmed in the social species for which biased adult sex ratios have been reported, we would then be faced with the interesting question of how species in five different spider families (Agelenidae, Dyctinidae, Eresidae, Theridiidae and Thomis-

idae) have solved the common problem of devising a mechanism by which to beat the odds imposed by the meiotic process (Williams 1979).

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APPENDIX

PROTOCOL TO OBTAIN SPIDER EMBRYO CHROMOSOME PREPARATIONS

1. **Fix the egg.** With a very fine needle, poke a small hole in the egg and with the egg so skewered, place it in a drop of fixative (3 parts absolute ethanol:1 part glacial acetic acid). We used electrochemically-sharpened tungsten wire needles. Limb buds, if present, appear as a series of small white lumps as the fixative enters the egg (a black background enhances visibility). Remove the tip of the needle from the egg, and press on the side of the egg so as to force the contents through the small hole. If the egg is young enough the contents can be squirted into a long thin string, which aids in rapid fixing and later in breaking the contents in small pieces. Tissue with nuclei is white; yolk without nuclei is yellowish; if there is much yolk then some can be discarded. Discard the empty chorion. Fix for 30 seconds.

2. **Squash the tissue.** Place the fixed contents of the egg in a small drop of 60% acetic acid on a microscope slide. With two very fine needles, break the tissue into small pieces. Place a cover slip on top, and squash the tissue flat. This squashing is perhaps the most critical step in the procedure: squashing too softly, sliding the cover slip sideways while squashing, and air bubbles should all be avoided.

3. **Remove the cover slip and let dry the tissue.** Freeze the slide on dry ice at least several minutes and flip the cover slip off with a razor blade. Wash off the acetic acid for 20 seconds in a bath of absolute ethanol. Let

the slide dry at least ten minutes. If needed, the slide can be left in this condition overnight or longer.

4. **Stain the tissue and make permanent the preparation.** After the slide has dried well, it can be stained and made permanent. No doubt many different stains could be used; we have used primarily a 3–4 minute bath of acetocarmine. After staining, the slide can be rinsed with appropriate solvents to prepare it for permanent mounting.